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SEMINARS IN Liver Disease

HEPATOCYTE TRANSPLANTATION AND LIVER-DIRECTED GENE THERAPY

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SEMINARS IN LIVER DISEASE

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Retroviral Vectors for Liver-directed Gene Therapy

GANJAM V. KALPANA, Ph.D.

ABSTRACT: Retroviruses are popular gene therapy vectors because they stably integrate the DNA copy of their genome into the host chromosome during their replication cycle. The widely used murine retroviral vector systems have two components: the transfer vector for the transgene carries all the cis-*acting elements necessary for the replication and efficient integration of the viral DNA; and the packaging cell line produces all the* trans-*acting proteins necessary for both structural and catalytic functions of the virus. Advances in design of retroviral vectors have resulted in greater degree of biosafety, expanded host range, and increased stability of the virus particles. Retroviral vectors have been widely used in the ex vivo gene therapy protocols to correct the liver diseases in a wide variety of species. In a limited number of applications, in vivo gene therapy has been achieved after the liver cells have been stimulated to regenerate. One major limitation of murine retroviral vectors is their inability to infect nondividing cells. This problem has been overcome by deriving vectors from lentiviruses (a class of retroviruses) that have the ability to infect both dividing and nondividing cells. The lentiviral vectors are derived from human immunodeficiency virus type 1 (HIV-1). Initial studies using lentiviral vectors for gene delivery to the liver in vivo show promising results. A highly crippled version of lentivirus has been generated by using producer cells in which the* trans-*acting components are expressed by several different coding elements and vectors that incorporate features of self-inactivation. These improvements should ensure biosafety of lentiviruses and make them useful in efficient delivery of therapeutic genes to nondividing differentiated tissues such as the liver.*

KEY WORDS: retroviral vectors, gene therapy, lentiviral vectors

Development of methods to deliver therapeutic genes to correct inborn or acquired deficiencies is a key aspect of gene therapy.[1] Of the several modes of gene delivery available today, use of recombinant viruses is the most powerful because it exploits the efficient machinery evolved by viruses to deliver nucleic acids into host cells.[2] Retroviruses, the best studied vectors for gene therapy, not only can deliver the nucleic acid genome, but also stably insert a complementary DNA copy of their genomic RNA into the host chromosome as an essential step of their life cycle.[1] This unique ability to efficiently integrate into host genome and to be faithfully transmitted to the progeny cells has made them the most popular gene therapy vectors. In addition to efficient entry and integration into cells, their broad tissue and host range, expression of transduced genes at high levels, ease of production at reasonably high titers, availability of producer cell lines (the packaging cell lines), and lack of adverse host immune response to retroviral vectors all add to their inherent advantage for use as vehicles for gene transfer.[3] Recent advances have resulted in lentiviral vectors, which have an unusual property that makes them suitable for noninvasive in vivo gene therapy of terminally differentiated tissues such as liver. This property is the ability of lentiviral vectors to infect and integrate into the genome of nondividing cells.[3] In this article, the principles underlying design, construction, and use of retroviral vectors for liver gene therapy will be discussed with a special emphasis on the lentiviral vectors.

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RETROVIRAL VECTORS

Salient Features of Retroviral Replication

A thorough understanding of retroviral replication has helped the design of efficient retroviral vectors. Therefore, a brief description of retroviral life cycle will be presented prior to the description of design of retroviral vectors.[4]

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The Structure of Viral RNA

Retroviruses are RNA viruses. The virus particle contains two identical copies of genomic RNA along with viral and cellular proteins (Fig. 1). There are three major open reading frames—*gag*, *pol*, and *env*—in the viral RNA that encode the structural and catalytic proteins required for various steps of viral replication. The viral RNA is flanked on either side by elements called long terminal repeats (LTRs). The 5′ end is flanked by regions R (for repeat) and U5, whereas the 3′ ends are flanked by regions U3 and R. The LTRs harbor the *cis*-acting promoter, upstream enhancers as well as polyadenylation signals essential for viral transcription. Additional *cis*-acting elements include signals (acceptor and donor sites) for proper splicing of mRNAs, the packaging sequence (ψ) that allows the viral RNA to be specifically packaged into the virus particle and primer-binding sites for the initiation of reverse transcription in the recipient cell (Fig. 1).

Early Events in Retroviral Replication

Viral entry into the host cell is mediated by a specific interaction of the envelope glycoproteins on the surface of virus particles with receptors on the host cell surface. The receptor–envelope interaction primarily determines host range. Murine amphotropic retroviruses have a broad host range, capable of infecting many different cell types from various species, including mouse and human, and have been the mainstay in the design of gene therapy vectors for humans. After entry into the host cell, the RNA genome is reverse-transcribed in the cytoplasm into double-stranded DNA. During the process of reverse-transcription, the U5 region is transferred to 3′ LTR and the U3 region is transferred to 5′ LTR in such a way that both LTRs possess U3, R, and U5 regions (Fig. 1).

The double-stranded DNA resulting from reverse transcription resides within a high molecular weight nucleoprotein complex called the preintegration complex (PIC), which includes the machinery necessary for integration of viral DNA. The transport of this large nucleoprotein complex from the cytoplasm into the nucleus of the infected cell is a prerequisite for integration. Unlike lentiviruses, the PICs of murine retroviruses (e.g., Moloney-murine leukemia virus [Mo-MuLV]) are incapable of crossing the nuclear membrane.[5] To access the host genome, they have to await the dissolution of nuclear envelope during mitosis. Thus, integration of murine retroviruses is dependent on cell division. Because integration is essential for their replication, murine retroviral vectors cannot infect nondividing cells and terminally differentiated tissues.

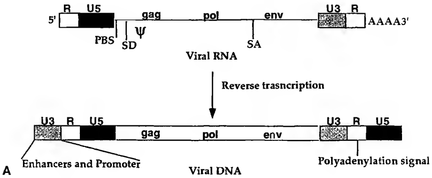
Replication-defective Retroviral Vectors

The most effective retroviral vectors are those that are capable of incorporating therapeutic genes, delivering them to the host cells via a single cycle of infection, and incapable of further spread. Such replication-defective viral vectors are derived by manipulating the viral genome and by replacing most or all of the viral genes by therapeutic transgenes.[1] The transgenes are replicated and integrated into the host genome along with the essential *cis*-acting elements of retroviral vectors. Generation of such virus particles with a mixed composition of partially defective genomic RNA encapsidated within functional viral proteins is possible because viral proteins necessary for processes associated with entry, reverse-transcription, and integration can be provided *in trans* by the helper cell lines, called the packaging cell lines. Because there is no need for viral protein synthesis during the early events of infection, the replication-defective virus produced in the packaging cell line can infect the recipient cell and integrate the vector DNA into the host genome without the *de novo* synthesis of viral proteins. Once integrated, it is no longer able to propagate in the absence of virus-specific genes. These replication-defective vectors are often called transducing viruses to distinguish them from replication-competent viruses.[1]

The replication-defective, transducing viruses are produced in two stages. First, the therapeutic gene of interest is cloned into the transducing vector DNA that is "crippled" or deleted of all the viral genes necessary for replication, such as *gag*, *pol*, and *env*, while retaining the "ψ" signals and other *cis* sequences necessary for the packaging of recombinant RNA molecule into the virus particle, and subsequent reverse-transcription and replication. Second, the recombinant viral DNA is transiently transfected into a packaging cell line that expresses the viral *gag*, *pol*, and *env*, proteins in trans via a second defective viral genome, called the helper virus, which is devoid of *cis*-acting sequences, including ψ sequences (Fig. 2).

Generating Retroviral Vectors with a High Degree of Biosafety

Early helper vectors contained simple deletions of the ψ region and were capable of producing all viral proteins, including Gag, Pol, and Env. Although the helper virus genome itself cannot be packaged, the recombination of helper virus with the transducing vectors leads to a possibility for regenerating a replication-competent recombinant (RCR).[6] This has lead to the design of multiply attenuated helper viruses. In some of the systems, the 3′-LTR of the helper virus, whose sole function was to provide polyadenylation signal, was re-



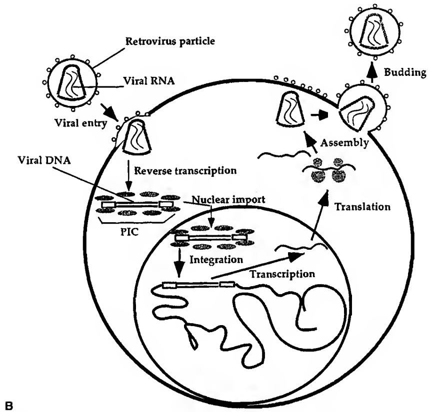


FIG. 1. Structure of genome and replication cycle of retroviruses. (A) Retroviral genomic RNA comprises three open reading frames for genes *gag, pol,* and *env* and is flanked at each end by LTRs. The 5′ LTR consists of regions R and U5; the 3′ LTR comprises regions U3 and R. Upon reverse transcription a double-standard (ds) DNA copy of the virus is formed that carries all the regions of viral RNA. In addition, each LTR end consists of three regions: U3, R and U5. Whereas the 5′ U3 region provides enhancers and promoter elements for the transcription of the integrated copy of the virus, the R region provides polyadenylation signals. ψ = packaging signal; PBS = primer binding site; SD = splice donor; SA = splice acceptor. **(B)** Retroviral life cycle. Viral RNA that is encapsidated in the particles enters the cytoplasm, where reverse transcription converts it into ds DNA. This DNA resides within a high molecular weight nucleo-protein complex called the preintegration complex (PIC). Upon entry of the PIC to the nucleus, integration of viral DNA to host DNA takes place. Subsequent steps of transcriprion of integrated viral DNA, translation, assembly, and budding releases the progeny virus particle.

placed with non retroviral polyadenylation signals, thus reducing the probability of recombination. A higher level of safety also has been ensured by using split genomes, where *gag/pol* and *env* are separated on two distinct helper genomes. This further reduces the probability of generating RCRs by increasing the number of crossovers required for recombination.

When split genomes are used, it is possible to pseudotype the transducing viruses by mixing the *gag/pol*-encoding genome from one virus with an *env*-

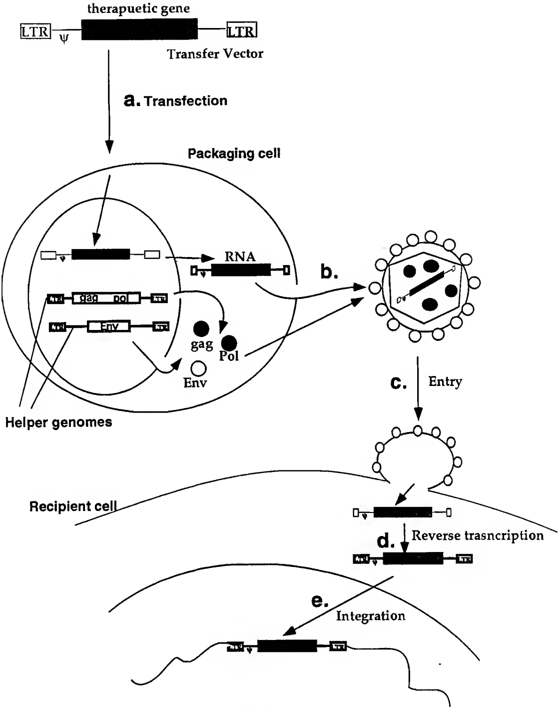


FIG. 2. Schematic representation of retroviral gene therapy vector system. (A) The gene of interest (therapeutic gene) is first cloned into the transfer vector carrying the *cis*-acting elements and the recombinant DNA is transfected into producer cells known as packaging cells. **(B)** Packaging cells carry helper genomes but, lacking the ψ signal, expresses the viral proteins that assemble along with the RNA from the trasnfer vector to produce viral particles. **(C)** The virus particles carrying vector RNA enters the cells. **(D)** The transfer vector along with the transgene is converted into DNA by reverse transcription. **(E)** Integration of the vector DNA results in the stable insertion of therapeutic genes in the recipient cells.

encoding genome from a different virus with different host range.[1] Pseudotyped viruses acquire the host range properties of the virus from which the envelope is derived but displays the replication capability of the virus from which the remaining sequences are derived. In general, the transducing viruses are pseudotyped with envelope from amphotropic retroviruses.

Self-inactivating Retroviral Vectors

In combination with the multiply attenuated helper vectors with split genome, an additional biosafety feature has been introduced by making the transfer vector handicapped or self-inactivating.[7–9] This is possible because of the modality of retroviral reverse-transcription, where the U3 region of both 5′ and 3′ LTRs of the viral DNA are derived from the U3 region of the 3′LTR of the viral RNA (Fig. 3). The U3 region carries promoters and enhancer elements necessary for the transcription of integrated provirus. A deletion introduced into the U3 region to remove enhancer and promoter elements at the 3′ end of the transfer vector gets transmitted to the 5′ region of the viral DNA in the recipient cell during reverse-transcription. When this viral DNA integrates, it lacks both 5′and 3′ U3 regions, and thus is not able to initiate transcription from the viral LTRs (Fig. 3). When using self-inactivating (SIN) retroviral vectors, the transgene is always expressed from an internal promoter. This design of SIN vectors also allows the use of cognate promoters as internal promoters because there will not be transcriptional interference from the 5′ LTRs. The second advantage of using SIN vectors is that the integrated proviral genome is not capable of activation of an adjacent cellular oncogene due to the lack of 3′promoters, thus increasing the biosafety measures.

An extensive list of all the available packaging cell lines carrying different helper viruses can be found elsewhere.[1]

Gene Therapy of Liver Using Retroviral Vectors

Standard murine retroviral vectors are well suited for ex vivo gene therapy applications of nondividing cells such as those in liver tissue. Hepatocytes from a variety of species, including mice, rats, rabbits, baboons, and humans, have been transduced with therapeutic genes using retroviral vectors ex vivo.[10–14] The integrating characteristic of retroviruses was exploited in the first successful long-term ex vivo gene therapy for a metabolic disease of the liver. In this study, hepatocytes were isolated from a resected liver lobe of low-density lipoprotein (LDL) receptor–deficient Watanabe heritable hyperlipidemic (WHHL) rabbits. The primary hepatocytes were established in culture and transduced with a MoMuLV-based vector expressing human LDLR.[10] Phenotypic correction of the transduced cells was demonstrated by reconstitution of the ability to internalize LDLs. Following retrovirus-mediated transduction, the cells were transplanted back into the donor rabbit by infusion into the portal vein.[10] Because the hepatocytes were autologous, no immunosuppression was needed. Long-term survival and function of the transduced transplanted cells was demonstrated by molecular analysis of liver biopsy specimens. The transplantation resulted in a 20 to 35% reduction of serum LDL cholesterol levels for the duration of the study (6 months).[10] Following this preclinical study, this ex vivo method was evaluated in a clinical study in four patients with familial hypercholesterolemia.[15,16] Although the reduction of serum LDL cholesterol was modest, and probably not therapeutically adequate, these studies showed the feasibility of transplanting transduced autologous liver cells in a clinical setting.[17]

Efficiency of ex vivo gene therapy using autologous primary hepatocytes is restricted by several factors. First, the number of hepatocytes that can be harvested from liver segments resected from a patient is limited. Second, the transduction of cultured primary hepatocytes, which divide only infrequently, is inefficient. Third, only a limited number of hepatocytes, representing 1 to 5% of the total hepatocyte mass, can be transplanted into the liver at one time.[18,19] In cases where the phenotypically normal transplanted cells have a definite survival advantage over the host hepatocytes, it is expected that the host liver eventually will be repopulated by a small number of transplanted hepatocytes. For example, in hereditary tyrosinemia type 1 (fumarylacetoacetate hydrolase deficiency) and progressive familial intrahepatic cholestasis type 3 (MDR3 abnormality) the life span of the host liver cells are markedly reduced. This should provide a selective proliferative pressure on the phenotypically normal transplanted cells, which should eventually replace the abnormal cells. These aspects are discussed more fully in the article by Grompe et al. in this issue.

To overcome some of the hurdles described above, two-stage retroviral transduction of hepatocytes has been performed in rats. Hepatocytes were harvested from a resected liver lobe of a bilirubin-UDP-glucuronosyltransferase–deficient (bilirubin-UGT) jaundiced Gunn rat.[20] As a first step, primary hepatocytes were transduced with a retroviral vector expressing a thermolabile mutant simian virus large T-antigen. This results in conditional immortalization of the hepatocytes. The cells proliferate in culture at 33°C. At physiologic temperatures, the mutant T-antigen is degraded, and the cells stop proliferating and express characteristics of differentiated hepatocytes.[21] During the proliferative phase of the cells, they were efficiently transduced using a second

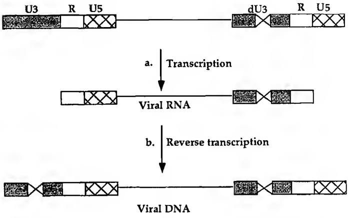


FIG. 3. Self-inactivating (SIN) vectors. These vectors carry a deletion of the essential enhancer and promoter elements in the U3 region (dU3) at the 3'end of the LTR of the transfer vector. **(A)** Transcription of the vector in the producer cell line results in viral RNA that only carries the deleted U3 region but not the 5' U3 region. **(B)** Reverse transcription of this RNA in the recipient cells transfers the deletions at the 3' U3 region to the 5' region of the resulting viral DNA. This deleted U3 region is not able to transcribe viral DNA, and hence is self-inactive.

recombinant retrovirus, expressing human bilirubin-UGT.[20] The phenotypically corrected transduced cells were cloned, expanded in culture, and transplanted into syngeneic Gunn rats in five different sessions. This resulted in approximately 30% reduction in serum bilirubin levels on a long-term basis. The transplanted immortalized hepatocytes were not tumorigenic, either in the Gunn rats or in immunodeficient (SCID) mice.

In some animal experiments, in vivo gene transfer to the liver has been accomplished using retroviruses.[21] Hepatocellular proliferation was induced in Gunn rats by 66% hepatectomy 24 hours before delivery of retroviral vectors. The inflow and outflow vessels of the liver were clamped and catheterized. The livers were perfused in situ with a recombinant retrovirus expressing human bilirubin-UGT. This resulted in sustained reduction of serum bilirubin levels for 18 months (duration of the study). Replacement of partial hepatectomy by other noninvasive approaches for the induction of hepatocellular proliferation is being investigated. These strategies include the administration of tri-iodothyronine, hepatocyte growth factor, lipopolysaccharides, and activators of Fas.[22–25]

PROGRESS IN DESIGN OF RETROVIRAL VECTOR SYSTEM

There are several limitations to the commonly used vector/packaging systems for in vivo gene therapy of liver and other nondifferentiated tissues. First, it is difficult to concentrate these viruses to achieve high titers as they are disrupted or ruptured during high-speed centrifugation. Second, amphotropic host range virus is inactivated by human serum. Third, they are unable to integrate in nondividing cells. Fourth, there is a possibility of transcriptional shut-off that occurs in vivo after prolonged periods. Recent advances in gene therapy strategies have resulted in overcoming many of these problems, which are discussed in the following sections.

Production of Highly Concentrated Retroviral Vector Particles

This has been achieved by pseudotyping the virus particles with envelope G glycoprotein of vesicular stomatitis virus (VSV), a class of RNA virus.[26,27] VSV-G protein was coexpressed in place of murine envelope protein, along with the helper virus that is devoid of *env* gene and transducing vector in the packaging cells. The resulting psuedotyped virus particles that had incorporated the VSV-G envelope possessed a wide host range of VSV and could be highly concentrated by centrifugation without loss of biological activity. Titers as high as 10^8 per ml could be achieved using this method.

Packaging Cell Lines with Inducible Synthesis of VSV-G

Initial studies used transient transfection to express VSV-G protein in the packaging cells because the constitutive expression of high levels of VSV-G in most cells is toxic. This significantly limits the application of pseudotyped viruses because only small amounts of the viruses could be produced at a given time. This toxicity problem has been overcome by developing human-derived packaging cell lines that express VSV-G under a tetracycline-inducible promoter.[28] In this case, the packaging cells harbor an altered gene encoding VSV-G protein under the tet^0 promoter, which is repressed by tet/VP16 protein in the presence of tetracycline. The tet/VP16 repressor is expressed from a second stably integrated construct and is inactivated upon the removal of tetracycline from the medium. In addition to these two constructs, the Gag-Pol proteins were expressed from a cytomegalovirus (CMV) promoter in the third construct. To generate retroviral vectors, the transducing vectors are first stably introduced into these packaging cells and cultured to obtain a desired cell density. Once the desired density of cells is

obtained, tetracycline-containing medium is removed and replaced by medium without tetracycline to inactivate the repressor. This results in the production of a large amount of VSV-G psuedotyped virus particles in the culture supernatants that can be concentrated 1000-fold, resulting in viral titers up to 10^9 infectious particles per ml.

Use of human-derived cells as packaging cell lines and incorporation of VSV-G proteins into retroviral vector has additional advantages. Unlike amphotropic envelopes, the VSV-G psuedotyped retroviral vectors were shown to be significantly more resistant to human serum.

LENTIVIRAL VECTORS

Unlike murine retroviruses, lentiviruses, a class of complex retroviruses, are capable of infecting both actively dividing as well as nondividing cells.[29] This property makes them most desirable for use in noninvasive in vivo gene therapy. Although the basic mode of replication of lentiviruses is similar to that of murine retroviruses, they differ in many aspects; hence, the life cycle of lentiviruses is discussed here, highlighting the differences followed by a description of latest advances in developing lentiviral vectors.

Salient Features of the Lentiviral Life Cycle

The best studied lentivirus is HIV-1. A crippled version of this virus has been developed into a lentiviral vector and has been used as a vehicle for in vivo gene delivery.[30] Like simple retroviruses, the genomic RNA of HIV-1 contains three major genes: *gag, pol,* and *env.* In addition to these, it also carries open reading frames for six accessory genes:*tat, rev, vpr, vpu, nef* and *vif*.[31,32] These accessory proteins are encoded by multiple exons in the viral genome and thus require multiple splicing of viral RNA for production (Fig. 4). The Tat and Rev proteins are important for the efficient gene expression at the transcriptional and posttranscriptional levels, respectively. Tat dramatically increases HIV-1 transcription by binding to TAR (transactivation response) sequences in the nascent RNA. TAR is a stem loop structure upon binding, to which Tat protein stimulates transcriptional elongation by RNA *Pol* II. Rev protein is essential for the export of unspliced and singly spliced viral transcripts. After transcription, the Rev protein binds to sequences in the viral RNA known as RRE (Rev response elements) and facilitates the nuclear export and cytoplasmic accumulation of viral transcripts.

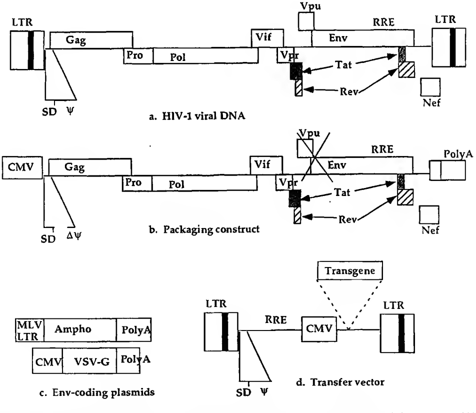


FIG. 4. Structure of HIV-1 viral DNA and the three plasmid based lentiviral vector system. (A) Structure of HIV-1 DNA indicating the open reading frames for major proteins and accessory proteins. **(B)** Packaging construct of the helper virus provides all the viral proteins except Env; there is a stop codon at the begining of Env and a deletion in the packaging signal (ΔΨ). **(C)** Envelope proteins, Ampho or VSV-G, are expressed from a separate plasmid vector. **(D)** The transfer vector carries all the *cis*-acting elements. It also carries an internal CMV promoter for the expression of transgene.

Both Tat and Rev are indispensable for the production of progeny virus particles. However, the remaining four accessory proteins Vif, Vpu, Vpr, and Nef are not essential for viral replication. Some of these, such as Vif and Nef, are required for in vitro viral replication in a cell type–specific manner. Some of them, such as Vpr and Nef, are thought to be necessary for pathogenesis in vivo. The mechanism of action of the above four accessory proteins are still not completely understood.[31,32]

The most striking feature of HIV-1 is its ability to replicate in nondividing cells. This property is due to the ability of the lentiviral PICs to get actively transported to the nucleus when the nuclear membrane is still intact.[29] The nuclear import of PICs is an ATP-dependent process and is mediated by the mutually exclusive nuclear localization signals in *vpr* and matrix protein (MA, which is encoded by *gag*). In addition, the integrase (IN protein, encoded by *pol*) is also thought to play an important role in nuclear localization. Thus, lentiviruses can get access to cellular DNA during all stages of the cell cycle, and integration of the viral DNA can proceed in the absence of active cell division.

Generating Lentiviral Vectors

Using retroviral vectors as models, lentiviral vectors have been generated by inserting the transgene between the LTRs and the packaging signal. The env protein of HIV-1 would limit the target cell specificity to T cells and macrophages. Therefore, similar to murine-retroviral vectors, the lentiviral vectors are pseudotyped with VSV-G to obtain broad host range. The VSV-G envelope significantly increases the stability of virus particles and hence allows the concentration of the lentiviral preparations by centrifugation as well. The use of VSV-G envelope does not induce cellular immune response or inflammations in vivo.

One of the first-generation lentiviral vectors developed is a three-plasmid–based system that includes a helper construct, an envelope-encoding construct, and the transducing vector (Fig. 4).[30] In this system, the helper construct lacks the viral 3′ and 5′ LTRs, ψ sequences, envelope-coding regions, and expresses the *gag, pol,* and accessory proteins from a CMV promoter. The *env*-coding plasmid expresses either an amphotropic envelope or a VSV-G envelope for psuedotyping. The transducing vector or transfer vector contains *cis*-acting sequences of HIV-1 necessary for reverse transcription and integration (3′ and 5′ LTRs and primer binding site), packaging sequences, splice signals, RRE for nuclear export, and unique restriction sites for cloning the transgene of interest. In addition to these *cis*-acting sequences, an internal CMV promoter and ribosomal entry site (RES) have been incorporated to facilitate the transcription of transgene in the absence of viral accessory proteins in the recipient cell (Fig. 4).

To generate the replication-defective virus particles, the three plasmids are transiently cotransfected into human 293T cells. All viral proteins are produced by the helper and envelope constructs, and the transducing RNA is produced by the transfer vectors, which are then assembled into virus particles and released into the supernatant. Virus particles in the culture supernatants can be concentrated by centrifugation if psuedotyped with VSV-G. These viral supernatants are then applied to desired tissues for gene delivery. As in retroviral vectors, proteins packaged in the virus particles are sufficient for the entry, reverse-transcription, and integration of transfer vector in the recipient cell. In the absence of viral proteins, the transfer vector is defective for viral replication, and only the transgene is expressed from an internal promoter.

Using Lentiviral Vectors for Liver Gene Therapy

The efficacy of the first-generation lentiviral vectors for in vivo liver gene therapy has been tested.[33] In this case, an *eGFP* (eukaryotic humanized green fluorescent protein) gene was cloned into the transfer vector upstream of a CMV promoter. Virus particles prepared as described above and pseudotyped with VSV-G protein were injected into the liver parenchyma of adult female nude rats. The transduction efficiency was determined by monitoring green fluorescence. About 3 to 4% of total liver tissue was transduced by a single injection of 1 to 3 $\times$ 10^7 infectious units of recombinant lentiviral vector. GFP was detected for a period of 6 months, the longest period tested after injection. No detectable inflammation was observed at the site of injection in the liver, indicating that there is no host mediated cellular immune response to lentiviral vectors. On the contrary, the MuLV-based vector used as a control did not show any transduction of liver cells in vivo. These results clearly indicate the usefulness of lentiviral vectors for in vivo liver gene therapy.

Progress in Design of Lentiviral Vectors

A major limitation in using lentiviral vectors for gene therapy is the biosafety consideration of introducing pathogen-derived sequences into humans. The concern is that the segregated *cis* elements of transfer vector could recombine with the *trans*-acting elements of the helper construct, or even more likely, with the endogenous retroviral sequences to regenerate RCR and present a risk to the recipient. The split-genome design of the three-plasmid vector described already ensures that such events are highly improbable because multiple crossover events need to take place in the correct order to regenerate RCR. Additionally, because the endoge-

nous retrovirus sequences present in the host genome do not have any nucleotide sequence homology to the lentiviral class of retroviruses, the probability of their recombination to generate RCR is minimal. Furthermore, one can also consider using simian or other lentiviruses as vectors, which will further reduce the possibility of homologous recombination. Improvements in the vector design have resulted in multiply attenuated vectors and self-inactivating vectors that should provide important additional safety features.

Multiply Attenuated Lentiviral Vectors

The second- and third-generation lentiviral vectors were generated by removing many of the accessory proteins from the vector system.[34] As discussed earlier, these accessory proteins are encoded by the helper construct. By introducing mutations or deletions, four of the accessory proteins that are not essential for vector-mediated transduction—Vpr, Vpu, Vif and Nef—were eliminated. It is known that the function of Tat is necessary in the packaging cells only for LTR-mediated transcription of transfer vector. However, if a part of the promoter present in the U3 region of LTR was replaced by a constitutive promoter such as CMV, the Tat dependency could be completely removed. Thus, five of the six accessory proteins can be eliminated. Moreover, the helper virus coding region was further split by removing the Rev coding region and providing it on a fourth plasmid.[35] Such highly crippled vectors both provide all the necessary *trans*-acting functions and are much safer. However, while using these vectors it is important to determine the requirement for the specific tissue in question. For example, it was noticed that efficient transduction in liver requires Vif and Vpu proteins, but they are not required for transduction in neuronal cells.[33] Although the mechanism of these differences is not known, it is thought that tissue-specific host cell proteins may complement the absence of these proteins in some cell types but not others.

Self-inactivating Vectors

Recently, SIN lentiviral vectors have been developed where most of the U3 (a 400-base pair) region of LTR, including the entire promoter region, has been removed.[36,37] In one case, only 53 nucleotides were left for (1) proper recognition and processing by integrase encoded by *pol* and (2) polyadenylation. This vector has all the functions necessary for transduction but is self-inactivated because deletion in the U3 region at the 3′end of the viral RNA is transmitted to the 5′LTR end of the viral DNA during reverse-transcription. Thus, the integrated viruses are nonfunctional due to extensive deletions in both the 3′ and 5′ U3 regions (Fig. 3).

PERSPECTIVES

Retroviral vectors offer great potential for gene therapy. Of several hundred clinical gene therapy trials currently ongoing, more than 45% are using retroviral vectors. Only a handful of clinical trials are directed to correcting liver diseases, including familial hypercholesteremia, liver metastasis, and acute liver failure (Wiley Clinical Trials Database). The major limitation in using murine-based retroviral vectors for liver-directed gene therapy, as discussed earlier, is their inability to infect nondividing cells. This problem can be solved by using lentiviral vectors because they can infect nondividing terminally differentiated tissues such as liver while retaining all the conveniences of retroviral vectors. The current improvements in lentiviral vectors eliminate the possibility of regenerating RCR and thus are excellent biosafety measures. However, because there is no animal model for HIV-1 infection, the efficacy and biosafety of lentiviral vectors can only be tested by clinical trials.

One problem associated with both lentiviral and retroviral vectors is the random integration of the transducing vector in the recipient cells. Because integration occurs without sequence specificity, there is the possibility that integration may activate an oncogene or inactivate a tumor suppressor gene. If integration can be targeted to a predetermined site in the chromosome either by modifying the integrase protein or the host factor that binds to it, then the danger of integrating into a harmful site can be eliminated.[38]

The ability to express the transgene from an internal promoter rather than LTRs of the vectors as in the case of lentiviral vectors provides great flexibility in controlling expression of the transgene. For example, liver-specific promoters or cognate promoters, if they are available, can be used to express a liver-specific gene. This ensures that the transgene is expressed at desired physiologic levels.

Unlike for retroviral vectors, no packaging cell lines are yet available for lentiviral vectors. Cell lines that constitutively express *trans*-acting factors eliminate the need for transient transfection of multiple constructs and will allow the generation of the large quantities of virus preparations necessary for in vivo gene therapy.

Another flexibility of retroviral vectors is that they can be pseudotyped with envelopes from other viruses, thus expanding the possibility of host range. Chimeric envelopes have been generated to incorporate part of the ligand for a cellular receptor. Viruses pseudotyped with such hybrid envelope proteins then interact with the cell surface receptor dictated by the ligand and mediate the cell type–specific infection by the retroviruses.[39,40] One can envision developing such hybrid envelopes for use in liver gene therapy. The lentiviral or retroviral vectors

could be pseudotyped with chimeric molecules carrying a hepatocyte-specific ligand. Such virus would specifically infect liver cells for efficient gene delivery.

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ABBREVIATIONS USED

CMV	cytomegalovirus
eGFP	eukaryotic humanized green fluorescent protein
HIV-1	human immunodeficiency virus type 1
LDL	low-density lipoprotein
LTR	long terminal repeat
Mo-MuLV	Moloney-murine leukemia virus
PIC	preintegration complex
RCR	replication-competent recombinant
RES	ribosomal entry site
RRE	Rev response element
SIN	self-inactivating retroviral
TAR	transactivation response
VSV	vesicular stomatitis virus
WHHEL	Watanabe heritable hyperlipidemic

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